DISTINCT DISTRIBUTION OF SPECIFIC MEMBERS OF PROTEIN 4.1 GENE FAMILY IN THE MOUSE NEPHRON

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Running title

4.1 family of proteins in the mouse nephron

Keywords

kidney — nephron — cytoskeleton — protein 4.1

Abbreviations

AE1: anion exchanger isoform 1; AQP2: aquaporin-2; BC: Bowman's capsule; CCD: cortical collecting duct; cTAL: cortical thick ascending limb of Henle's loop; DCT: distal convoluted tubule; FITC: fluorescein isothiocyanate; HA: hemagglutinin; His: histidine; IMCD: inner medullary collecting duct; mTAL: medullary thick ascending limb of Henle's loop; NHE: sodium proton exchanger; OMCD: outer medullary collecting duct; PBS: phosphate buffered saline; PCR: polymerase chain reaction; PCT: proximal convoluted tubule; RACE: rapid amplification of cDNA ends; RT: room temperature; SAB: spectrin-actin binding; TAL: thick ascending limb of Henle's loop; Tm: melting temperature.

Abstract

DISTINCT DISTRIBUTION OF SPECIFIC MEMBERS OF PROTEIN 4.1 GENE FAMILY IN THE MOUSE NEPHRON. Background: Protein 4.1 is an adapter protein which links the actin cytoskeleton to various transmembrane proteins. 4.1 proteins are encoded by four homologous genes, 4.1R, 4.1G, 4.1N, and 4.1B, which undergo complex alternative splicing. Here we performed a detailed characterization of the expression of specific 4.1 proteins in the mouse nephron. Methods: Distribution of renal 4.1 proteins was investigated by staining of paraformaldehyde fixed mouse kidney sections with antibodies highly specific for each 4.1 protein. Major 4.1 spliceforms, amplified from mouse kidney marathon cDNA, were expressed in transfected COS-7 cells in order to assign species of known exon composition to proteins detected in kidney. Results: A 105kDa 4.1R spliceform, initiating at ATG-2 translation initiation site and lacking exon 16, but including exon 17B, was restricted to thick ascending limb of Henle's loop. A 95kDa 4.1N spliceform, lacking exons 15 and 17D, was expressed in either descending or ascending thin limb of Henle's loop, distal convoluted tubule and all regions of the collecting duct system. A major 108kDa 4.1B spliceform, initiating at a newly characterized ATG translation initiation site, and lacking exons 15, 17B, and 21, was present only in Bowman's capsule and proximal convoluted tubule (PCT). There was no expression of 4.1G in kidney. Conclusion: Distinct distribution of 4.1 proteins along the nephron suggests their involvement in targeting of selected transmembrane proteins in kidney epithelium and therefore in regulation of specific kidney functions.

Introduction

One of the major functions of the kidney is to ensure body homeostasis by balancing water and metabolite reabsorption and excretion, these events taking place in specialized regions of the nephron (1). At the cellular level, a large part of this molecular traffic occurs through specific transporters whose activity is mostly controlled by various hormones and neuromediators (2). Over the past ten years, there has been increasing evidence for regulation of the activity of ion transporters by the cytoskeleton (3-5). Members of the protein 4.1 superfamily, such as ezrin, known to act as adapter proteins between the actin cytoskeleton and transmembrane proteins, have been reported to play a key role in regulation of activity and trafficking of kidney transporters, such as the sodium proton exchanger NHE3 (6-11). The functional impact of such interactions has been further emphasized by the observation that NHEs are involved in organization of cortical cytoskeleton and in control of cell shape independently of ion transport activity (12). An important discovery is that NHE regulation differs between proximal and distal regions of the nephron (8). It has been proposed that this could result from differences in protein scaffolds involved in cytoskeleton architecture. Such a hypothesis is supported by the observation that the cytoskeletal proteins ankyrin and fodrin display differential distribution along the nephron (13-15). This complex spatial distribution likely results from the fact that each of these cytoskeletal proteins is actually expressed as a panel of isoforms encoded by highly related but distinct genes. For example, ankyrin is expressed as various isoforms encoded by three homologous genes (16,17). Expression of one of these ankyrin genes, Ank3, is mostly restricted to epithelial tissues, such as kidney (14,16-18). An Ank3 isoform, AnkG190, has been reported to interact with a key kidney transporter, Na+,K+-ATPase (19). Taken together, these studies have infused new interest into evaluating the functional relevance of cytoskeletal proteins in kidney. Surprisingly, characterization of renal protein 4.1 has been overlooked so far, despite the fact that this protein, functionally related to ankyrin (17, 20) and ezrin (21), is known to interact with key ion transporters, such as the anion exchanger AE1 (22-24).

Protein 4.1 was originally characterized in red blood cells where it plays a key role in the regulation of mechanical stability (20). Subsequent characterization in nucleated cells demonstrated that protein 4.1 adopts a broad cellular distribution (25), which results from the combination of two factors. First, the protein 4.1 gene undergoes complex alternative splicing of its pre-mRNA, resulting in translation of numerous spliceforms from a single gene (26,27). Second, we, and others, have recently characterized three additional protein 4.1 genes, 4.1G (28,29), 4.1N (30,31), and 4.1B (32,33), which are very homologous to the original red blood cell protein 4.1 gene, renamed 4.1R in order to distinguish it from these new genes. Importantly, the splicing of protein 4.1R is tissue-specific, developmentally-regulated, and also influenced by cellular interactions with the extracellular matrix (34). As expected from its broad cellular distribution, protein 4.1R interacts with a growing list of binding partners located throughout the cell (20,35). The functional impact of potential interactions between protein 4.1R, or its homologues, and ion transporters and tight junction proteins (36), has yet to be defined.

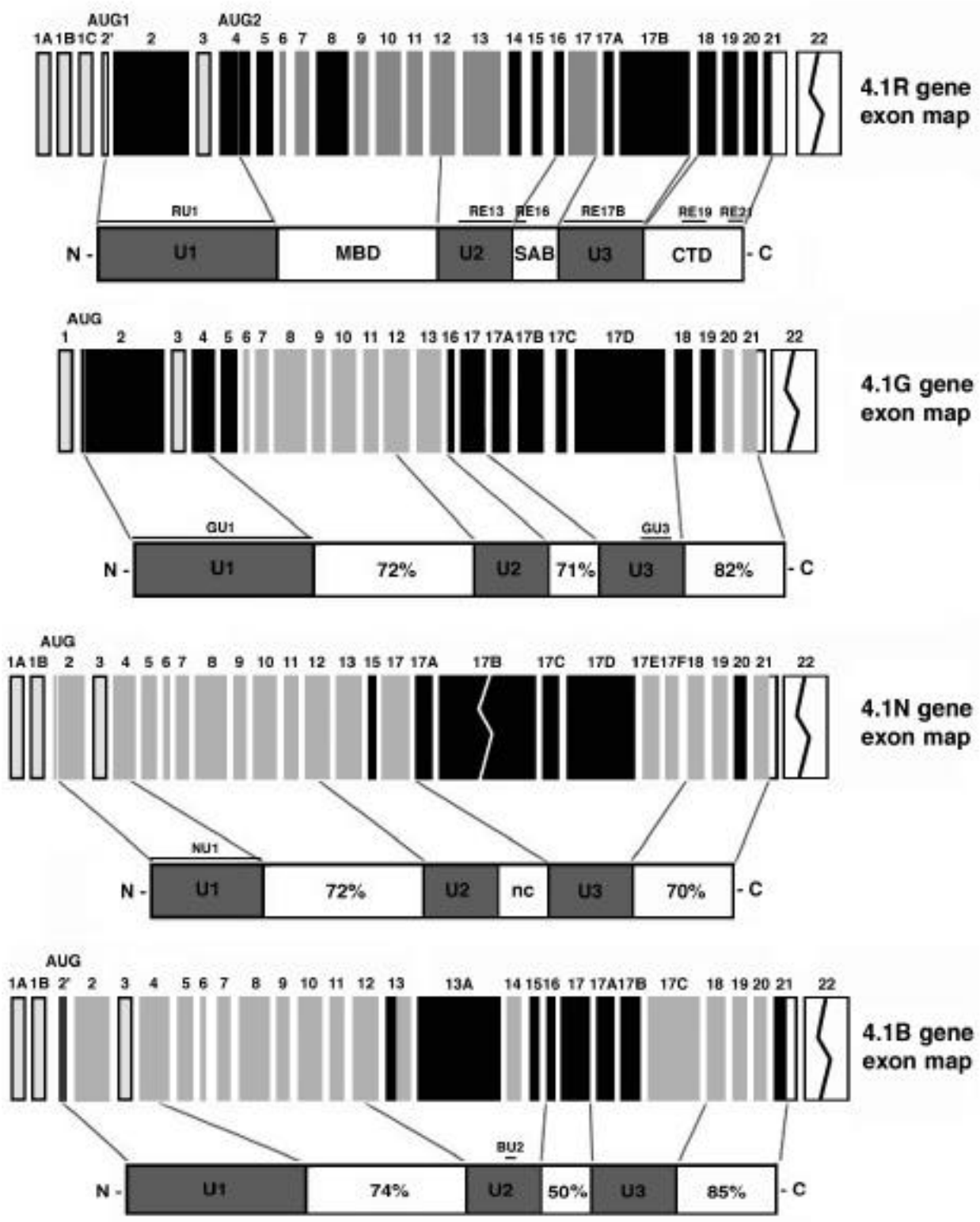
As a first and necessary step to investigate the functional relevance of protein 4.1 in kidney epithelium, we performed a detailed characterization of the expression of the four 4.1 proteins in this tissue. Immunohistochemistry studies show specific tissue and cellular distributions for each renal 4.1 protein. Amplification of kidney 4.1 cDNAs reveals that each 4.1 protein exists as various spliceforms and, consistent with this observation, renal 4.1 proteins are expressed as various protein isoforms *in vivo*. The findings outlined here establish a foundation for identifying renal 4.1 protein binding partners, and for determining the roles of 4.1 proteins in kidney structure and function.

Material and Methods

Reagents - All chemicals used in this study were of reagent grade.

Cell lines and mice - Monkey kidney fibroblast (COS-7) cell line was from American Type Cell Culture (Manassas, VA). 129/SV strain wild type mice, or 4.1R null mice generated by us as previously described (37), were bred on site.

Cloning of recombinant histidine (His)-tagged 4.1 proteins - cDNAs encoding full length mouse 4.1R (initiated at ATG-1 and containing exon 17B-encoded peptide, see Figure 1), mouse 4.1G (29), mouse 4.1N (31), and mouse 4.1B (33), were cloned into pET31b(+) vector (Novagen, Madison, WI). His-tagged recombinant 4.1 proteins, expressed in BL21 Gold (DE3) *E. Coli* (Stratagene, La Jolla, CA), were purified according to the manufacturer's instructions (Novagen), with the following modification. Because of their insolubility, proteins were extracted in binding buffer supplemented with 6M urea. After affinity purification, proteins were dialyzed against phosphate buffered saline (PBS).


AUG1
AUG2
1A 1B 1C 2' 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 17A 17B 18 19 20 21 22
4.1R gene exon map
RU1
RE13
RE16
RE17B
RE19
RE21
N -
U1
MBD
U2
SAB
U3
CTD
- C
AUG
1 2 3 4 5 6 7 8 9 10 11 12 13 16 17 17A 17B 17C 17D 18 19 20 21 22
4.1G gene exon map
GU1
GU3
N -
U1
72%
U2
71%
U3
82%
- C
AUG
1A 1B 2 3 4 5 6 7 8 9 10 11 12 13 15 17 17A 17B 17C 17D 17E 17F 18 19 20 21 22
4.1N gene exon map
NU1
N -
U1
72%
U2
nc
U3
70%
- C
AUG
1A 1B 2' 2 3 4 5 6 7 8 9 10 11 12 13 13A 14 15 16 17 17A 17B 17C 18 19 20 21 22
4.1B gene exon map
BU2
N -
U1
74%
U2
50%
U3
85%
- C

Protein 4.1 antibodies - Antibodies were raised against recombinant proteins or peptides encoded by unique regions of mouse 4.1R, 4.1G, 4.1N, or 4.1B (Figure 1). Mouse 4.1R, 4.1G, and 4.1N antibodies were raised in rabbit against recombinant histidine (His)-tagged proteins corresponding to mouse 4.1R exon 13-encoded peptide, mouse 4.1R exon 17B-encoded peptide, mouse 4.1G unique region U1 (29), part of mouse 4.1G unique region U3 (38), or mouse 4.1N unique region U1 (31). Mouse 4.1B antibody was raised in goat against a 20mer peptide within the unique region U2 (33). We also used four antibodies raised in rabbit against either human 4.1R unique region U1, a 21mer peptide encoded by human 4.1R exon 16 (39), a 34mer peptide encoded by human 4.1R exon 19 (39), or a 20mer peptide corresponding to the very last amino acids encoded by human 4.1R exon 21 (39). All antibodies, except for mouse 4.1R exon 17B-encoded peptide antibody, were affinity purified. Specificity of protein 4.1 antibodies was assessed by comparing their reactivity against 100ng of each of the four recombinant His-tagged full length mouse 4.1 proteins described in the previous section. Processing of the corresponding Western blots is described in the Western blotting section.

Immunohistochemistry - Mouse kidney sections were processed for immunohistochemistry as previously described (40) with some modifications. Mice were anesthetized, perfused with 10 ml perfusion buffer in order to exsanguinate tissues, then with 10ml PBS containing 4% paraformaldehyde in order to fix tissues *in vivo*. Kidneys were harvested, cryopreserved in PBS + sucrose, and flash frozen in liquid nitrogen. 10µm thick kidney cryosections were stained overnight at 4°C with specific 4.1 antibodies diluted 1/25 (anti 4.1R E19 antibody) or 1/10 (anti 4.1N U1 and 4.1B U2 antibodies), then for 2h at RT with either anti rabbit IgGs (4.1R and 4.1N) or anti goat IgGs (4.1B) coupled to Cy3 (Jackson Laboratories, West Grove, PA) diluted 1/200. In experiments where both 4.1 and AQP2, a specific marker for principal cells in collecting duct (41), were detected, and since antibodies for both antigens were rabbit polyclonal antibodies, kidney sections were first stained with 4.1 antibody as described above, then incubated for 1h at RT with rabbit IgGs diluted 1/10,1h at RT with anti rabbit Fab fragments (Jackson Laboratories, ME) diluted 1/50, then stained overnight at 4°C with anti AQP2 antibody (42) diluted 1/100 followed by secondary anti rabbit IgGs coupled to Alexa 488 (Molecular Probes, Eugene, Oregon) diluted 1/100. In experiments where both 4.1 and the Tamm-Horsfall protein, a specific marker for the thick ascending limb of Henle's loop (43), were detected, kidney sections were first stained with 4.1 antibody then with Tamm-Horsfall protein antibody (Valbiotech, Paris, France) diluted 1/50, followed by anti goat IgGs coupled to FITC (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted 1/50. Sections were mounted and observed with a Zeiss LSM 510 confocal microscope (Zeiss Inc., Germany).

Preparation of tissue extracts - 129/SV strain wild type or 4.1R null mice were perfused with 10ml PBS containing 1mM EGTA, 1mM difluoroisopropylfluorophosphate, and a cocktail of protease inhibitors (Sigma-Aldrich, St Louis, MO) (perfusion buffer) in order to minimize red blood cell contamination in tissue extracts. Kidneys, spleen and brain were homogenized in 5ml cold lysis buffer (perfusion buffer containing 1% IGEPAL CA-630 (Sigma Aldrich)). Aliquots were kept for protein assay, while lysates were denatured in Tris-glycine sample buffer containing sodium dodecyl sulfate and 100mM dithiotreitol and used for Western blotting.

Western blotting – Samples were run on 8% Tris-Glycine gels (Invitrogen, Carlsbad, CA) and gels were transferred onto Immobilon-P polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Membranes were blocked for 1h at room temperature (RT) in blotting buffer (PBS + 1% Tween-20 + 4% non fat dry milk + 1% BSA + 1% donkey serum), then incubated overnight at 4°C with primary 4.1 antibodies diluted at 0.1-0.3µg/ml (mouse 4.1R exon 17B peptide antibody diluted 1/3000) in blotting buffer. After extensive washes in PBS + 1% Tween-20, membranes were re-blocked for 15 min in blotting buffer without donkey serum, then incubated for 1h at RT with either biotinylated donkey anti rabbit IgGs (Amersham Pharmacia Biotech., Piscataway, NJ), for 4.1R, 4.1G, and 4.1N blots, or biotinylated donkey anti goat IgGs, for 4.1B blot, diluted 1/500 in blotting buffer without serum. After extensive washes, membranes were re-blocked for 15min

with complete blotting buffer, and incubated for 1h at RT with streptavidin coupled to horseradish peroxidase (Amersham Pharmacia Biotech.), diluted 1/40,000 in blotting buffer. After extensive washes, membranes were processed with chemiluminescent Renaissance reagent (NEN Dupont, Boston, MA). Blots used to assess antibody cross-reactivity were processed as above except that recombinant His-tagged full length mouse proteins 4.1R, 4.1N, 4.1B and 4.1G were run on 6% Tris-Glycine gels. In order to confirm purity and equal loading of the recombinant proteins, recombinant proteins normalized after protein assay, were run on a gel and stained with Gelcode® blue stain reagent (Pierce, Rockford, IL). Control blots were probed with either pre-immune rabbit (for 4.1R, 4.1N and 4.1G antibodies), or goat (for 4.1B antibody) serums. In experiments designed to assess specificity of detection of renal 4.1 proteins, blots were processed as described above except that primary 4.1 antibodies were pre-adsorbed or not prior to dilution in blotting buffer and incubation with PVDF membranes. Briefly, anti 4.1R E13, anti 4.1R E19, anti 4.1N U1 and anti 4.1B U2 antibodies were incubated for 2h at 4°C with either PBS or PBS containing a 10 molar excess of either recombinant His-tagged full length mouse 4.1R for anti 4.1R E13 and anti 4.1R E19 antibodies, full length mouse 4.1N for anti 4.1N U1 antibody, or full length mouse 4.1B for anti 4.1B U2 antibody (4.1G was not tested in these latter assays because of its lack of expression in kidney).

Protein assay - Protein assays were performed using DC protein reagent (Bio-Rad, Hercules, CA) with BSA as a standard.

5' rapid amplification of cDNA ends (RACE) and 3' RACE of renal 4.1 genes - Splicing events taking place either in the 5'half or in the 3'half of each 4.1 protein gene were investigated by amplifying mouse kidney marathon cDNA by polymerase chain reaction (PCR) according to the manufacturer's instructions (BD Biosciences). The AP1 primer, specific for the adapters flanking marathon cDNAs, was used in combination with either reverse primers (5' RACE), or forward primers (3'RACE), specific for each protein 4.1 gene (Table II). The 4.1 gene specific primers were chosen in the MBD region, a region subject to very few alternative splicing events.

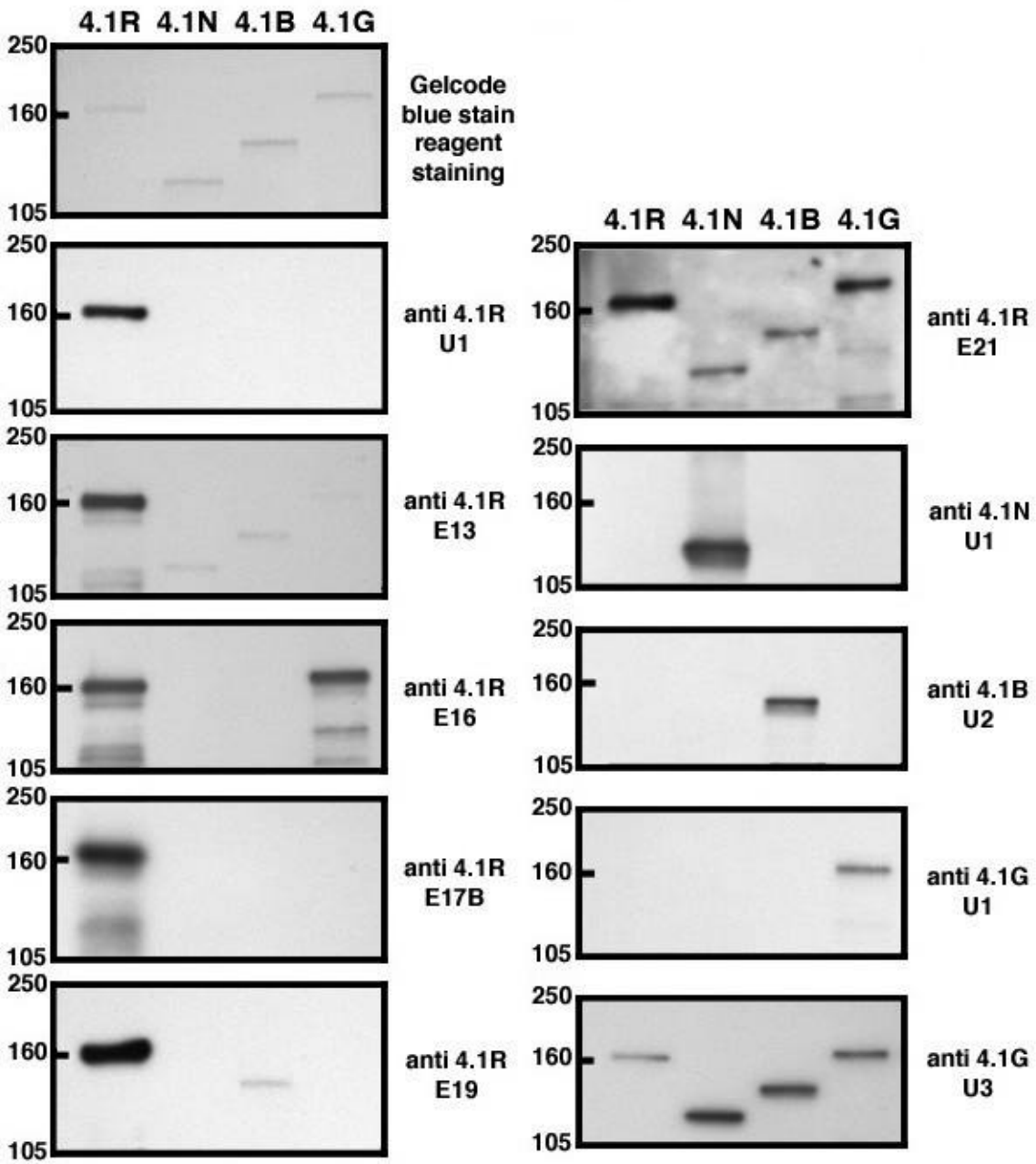
Amplification of renal 4.1 full length coding regions - Full length 4.1 protein spliceoforms were amplified by PCR from mouse kidney marathon cDNA, using pairs of protein 4.1 gene-specific primers designed in light of the information obtained from 5'RACE and 3'RACE experiments. Forward primers bear a Hind III restriction site, while reverse primers bear either a Xho I (in the case of 4.1R and 4.1B) or a Sal I (in the case of 4.1N and 4.1G) restriction site in order to allow subsequent cloning of PCR products into C-terminal hemagglutinin (HA)-tagged mammalian expression vector pCDNA3 (Invitrogen) as previously described (25,33,44).

COS-7 cell transfection - COS-7 cells were transfected with cDNAs encoding major renal HA-tagged 4.1 protein spliceoforms as previously described (25,33,44) with minor modifications. Briefly, cells grown in 100mm cell culture dishes, were transfected with 12µg of DNA and 36µl of lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). 24h after transfection, cells were washed twice with PBS, then denatured and processed for Western blotting as described above.

Results

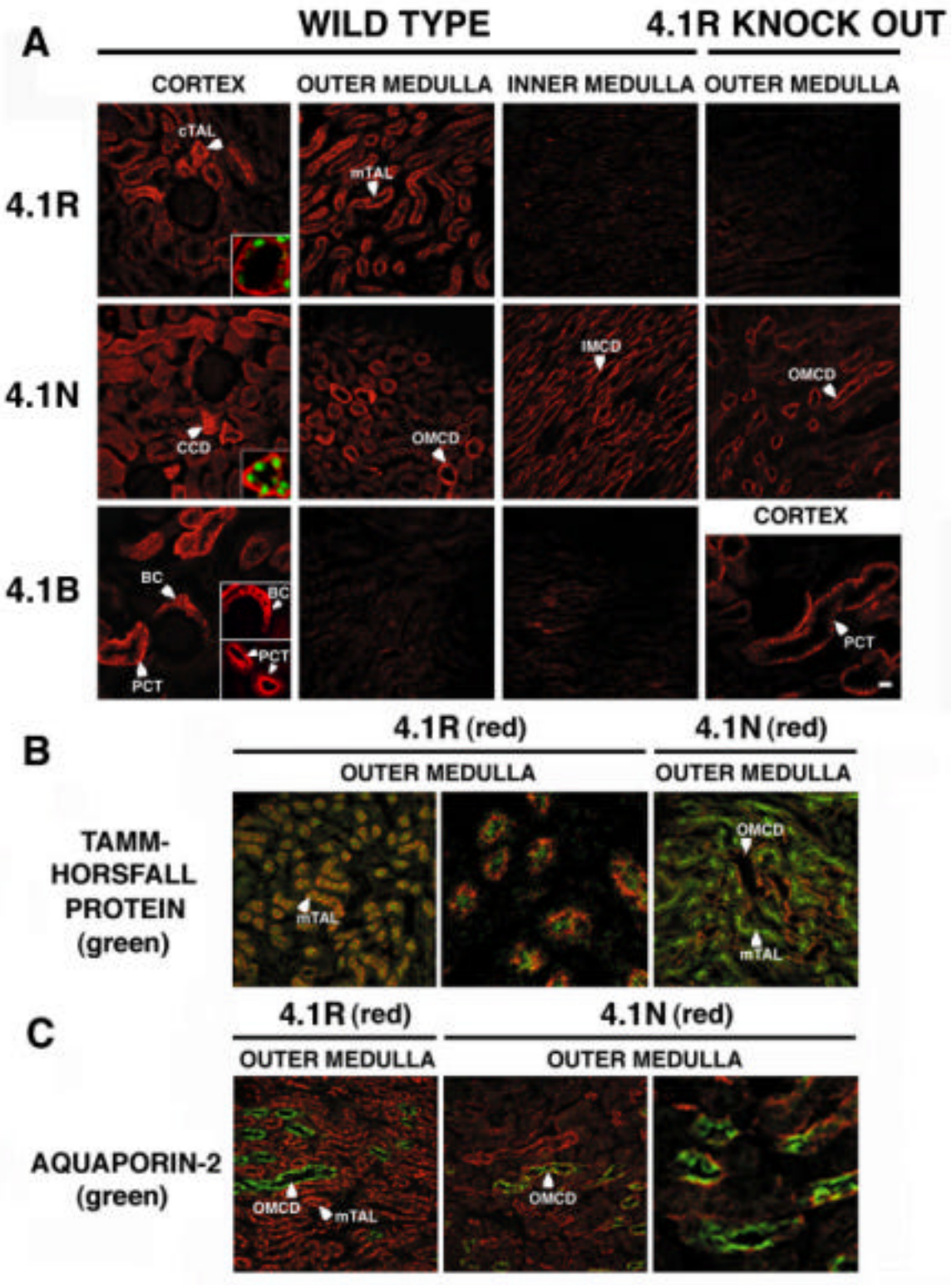
Distinct distribution of 4.1 proteins in kidney — As a first step towards characterizing 4.1 proteins in kidney, we investigated their distribution within the tissue by staining mouse kidney sections with various protein 4.1 antibodies. Given the very high identity of amino acid sequence

between the four 4.1 proteins, we raised antibodies against unique regions of each 4.1 protein to ensure specificity of protein detection (Figure 1). Antibody specificity was investigated by probing each antibody against the four recombinant full length mouse 4.1 proteins expressed in *E. Coli*. Most antibodies reacted predominantly with their corresponding recombinant 4.1 protein (Figure 2 and Table I). Three antibodies showed strong cross-reactivity: anti 4.1R E16 antibody with 4.1G, anti 4.1R E21 antibody with 4.1N, 4.1B and 4.1G, and anti 4.1G U3 antibody with 4.1R, 4.1N and 4.1B, respectively (Figure 2). Because of their extensive lack of specificity, anti 4.1R E21 and anti 4.1G U3 antibodies were not used in the study. Three antibodies showed weak cross-reactivity: anti 4.1R E13 antibody with 4.1N, 4.1B and 4.1G, anti 4.1R E19 antibody with 4.1B and anti 4.1N U1 antibody with 4.1R, 4.1B and 4.1G, respectively (Table I). Such weak signals could only be detected after long exposure of the blots leading to a saturation of the signal obtained with the protein reacting strongly with the antibody. Therefore, as extensively shown in this study, these weak cross-reactivities did not end up being very relevant.



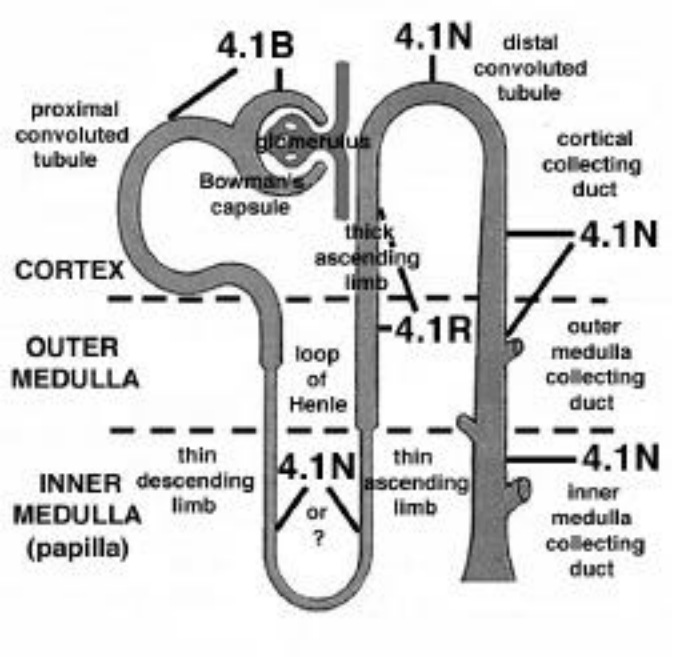
Having investigated antibody specificity, we probed paraformaldehyde-fixed mouse kidney sections with selected antibodies highly specific for each 4.1 protein, i.e anti 4.1R E19, anti 4.1N U1, anti 4.1B U2 and anti 4.1G U1 antibodies. Kidney 4.1R showed strong expression in cortex and outer medulla, but poor expression in inner medulla (Figure 3A, first row, three left panels). The protein showed obvious baso-lateral expression (Figure 3A, first row, left panel, inset). Specificity of detection of protein 4.1R was confirmed by the lack of signal in 4.1R null kidney, in contrast to the presence of staining in wild type kidney (Figure 3A, compare left and right panels of first row for an example in outer medulla). This observation supported that anti 4.1R E19 antibody was unable to interact with 4.1B in kidney sections, despite its weak cross-reactivity with recombinant 4.1B (Table I). The distribution of 4.1R was the same as that of Tamm-Horsfall protein (Figure 3B; left

panel and middle panel for magnification), a specific marker for TAL (43). In contrast, 4.1R did not co-localize with water channel aquaporin-2 AQP-2 (Figure 3C, left panel), a specific marker for principal cells in collecting duct (41). Kidney 4.1N showed strong expression in cortex, outer medulla and inner medulla (Figure 3A, second row, three left panels). The protein was found in the thin limb of Henle's loop, distal convoluted tubule (DCT) and all regions of the collecting duct system, i.e. cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). Specific detection of 4.1N was supported by the fact that protein expression was similar in wild type and 4.1R null kidney (Figure 3A, compare left and right panels of second row for an example in outer medulla). This observation was in accordance with the absence of cross-reactivity of anti 4.1R E19 antibody with recombinant 4.1N (Figure 2), and the very weak cross-reactivity of anti 4.1N U1 antibody with recombinant 4.1R (Table I). Like 4.1R, 4.1N was distinctly expressed at the baso-lateral pole (Figure 3A, second row, left panel, inset). However, unlike 4.1R, 4.1N did not localize in the same structures as Tamm-Horsfall protein, i.e. in TAL (Figure 3B, right panel). Co-staining of 4.1N and water channel AQP-2 revealed that 4.1N was present in collecting duct, with protein expression in both intercalated and principal cells (Figure 3C; middle panel and right panel for magnification). Thus, 4.1R and 4.1N have distinct distributions within the kidney. Interestingly, the most restricted distribution was observed for 4.1B. 4.1B showed strong expression in cortex but no expression in either outer or inner medulla (Figure 3A, third row). 4.1B expression was similar in wild type and 4.1R null kidney cortex (Figure 3A, third row, compare left and right panels), emphasizing the specificity of detection of 4.1B as expected from the absence of cross-reactivity of the anti 4.1B U2 antibody with 4.1R, 4.1N and 4.1G (Figure 2). 4.1B expression appeared diffuse in Bowman's capsule (BC), where it seemed to be restricted to parietal cells (Figure 3A, third row, left panel). 4.1B was present mostly in the baso-lateral pole of PCT (Figure 3A, third row, left panel), although some PCTs showed strong apical staining (Figure 3A, third row, left panel, lower inset). Using our antibodies, we were unable to detect 4.1G by immunostaining, an observation confirmed by Western blotting experiments (see section below).



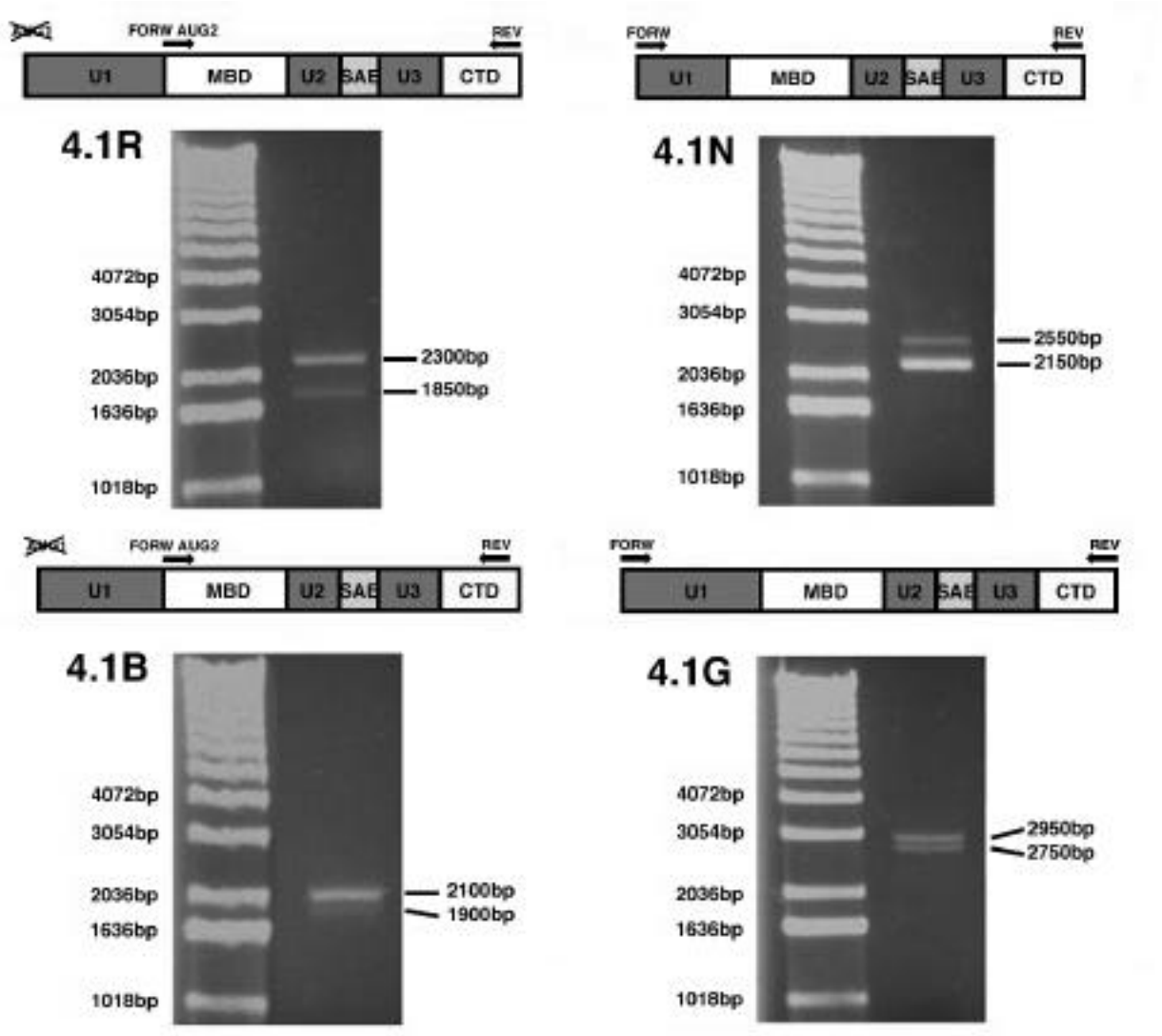
These data clearly show distinct distributions of 4.1 proteins along the various segments of the nephron, as summarized in Figure 4. The dramatic heterogeneity in the distribution of 4.1 proteins within the kidney epithelium strongly supported the hypothesis that each 4.1 protein might play

distinct roles in kidney structure and function. However, it became clear that it was critical to decipher the structure of renal 4.1 proteins prior to implementing such functional investigations.



Multiple 4.1 protein cDNAs are expressed in kidney — In order to deduce the primary structure of the major renal 4.1 proteins, we used PCR techniques to amplify 4.1 sequences from mouse kidney marathon cDNA (see Methods section). Due to the complex structure of the 4.1 genes, including the potential for multiple 5' and 3' ends (26-27, 30, 33), accurate characterization required both 5' and 3' RACE experiments prior to amplification of full length coding regions.

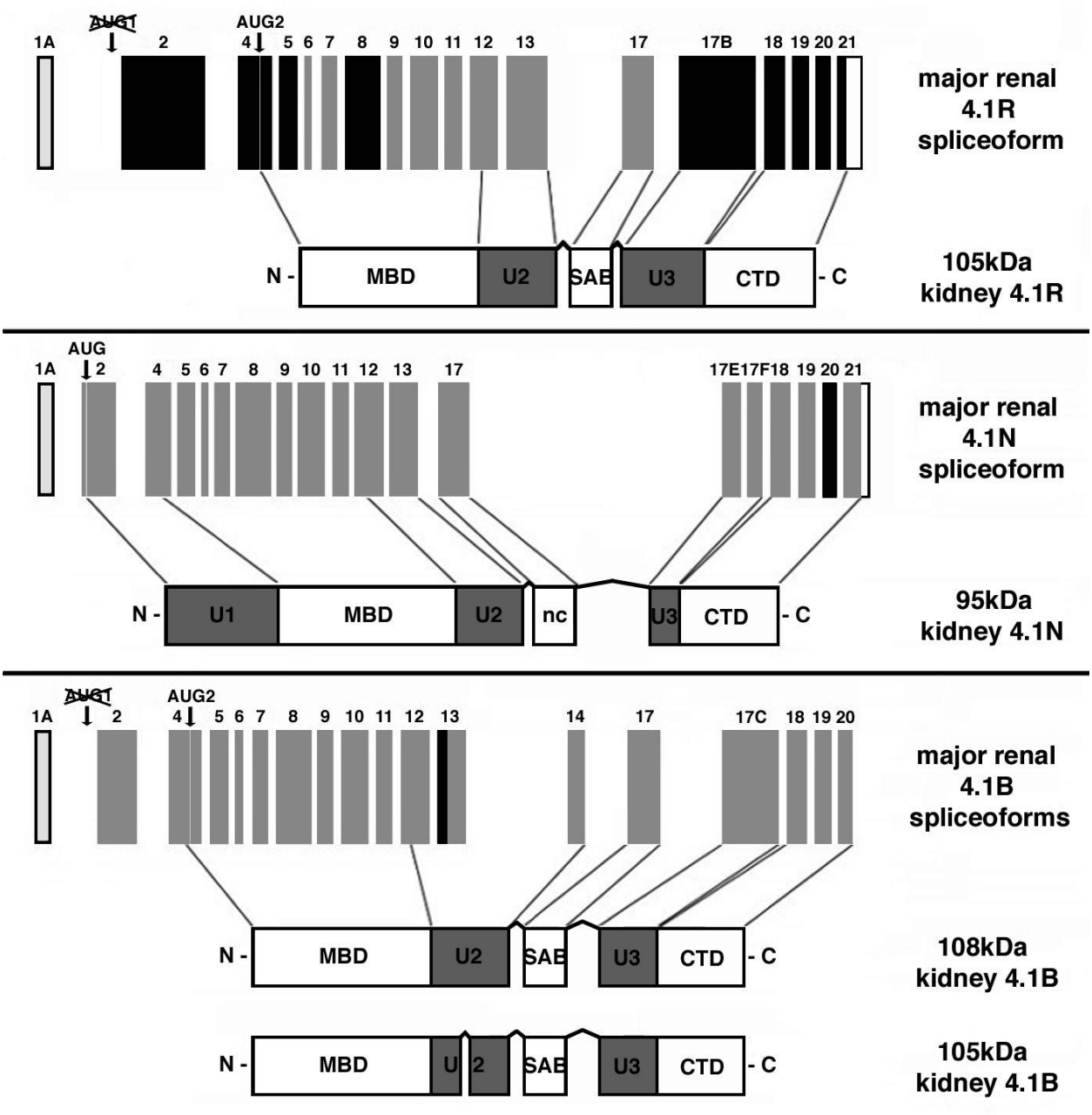
Mouse kidney 4.1R - 5'RACE experiments, using primer AP1 and a mouse 4.1R specific reverse primer (Table II), revealed that kidney 4.1R lacked exon 2', a 17bp exon which includes the upstream translation initiation site ATG-1 (26,27). Thus, translation of kidney 4.1R was only initiated at the downstream ATG-2 site located in exon 4 (26,27). 3'RACE experiments, using primer AP1 and a mouse 4.1R specific forward primer (Table II), did not detect any unexpected structures in the 3' region of the 4.1R transcripts. Amplification of full length kidney 4.1R coding region, using a forward primer in the ATG-2 region in exon 4, and a reverse primer specific for the end of the coding region encoded by exon 21, resulted in amplification of two PCR products of 2300bp and 1850bp (Figure 5). The predominant 2300bp PCR product included exon 17B, a 450bp exon specifically expressed in epithelial tissues (34). Sequence analysis of individual subclones derived from this PCR product further showed that it was actually a mixture of two spliceforms differing by either inclusion or exclusion of exon 16, a 63bp exon located within the spectrin actin binding (SAB) domain (26,27). Most of the clones excluded this exon (28 out of 33 clones). These two species could not be visually discriminated within the 2300bp PCR product due to the small size of exon 16. The minor 1850bp PCR product excluded exon 17B. This product also comprised a mixture of two spliceforms including or excluding exon 16, with a majority of the clones (15 out of 19 clones) once again lacking this exon. All clones lacked exons 14 and 15, and all but one clone lacked exon 17A (45,46). Taken together, these data led us to conclude that the major kidney 4.1R isoform was initiated at ATG-2, and that it included all exons of the coding region except exons 2', 14, 15, 16, and 17A (Figure 6).



Mouse kidney 4.1N — Neither 5'RACE nor 3' RACE experiments, using primer AP1 and either a mouse 4.1N specific reverse primer or a mouse 4.1N specific forward primer, respectively (Table II), revealed any splicing event which could alter kidney 4.1N translation at the very 5' or 3' end of the coding region. Amplification of full length mouse kidney 4.1N coding region generated two PCR products of 2550bp and 2150bp (Figure 5). The predominant 2150bp species lacked first, a 36bp exon located in unique region U2 (Figure 1) and sharing high homology with mouse 4.1B exon 15 (33), and second, a 408bp exon in unique region U3 assigned to exon 17D (Figure 1). These two exons were present in the minor 2550bp species. A minor splicing event consisted of exclusion of exon 20 (1 out of 8 clones). Taken together, these data showed that kidney 4.1N was differing from brain 4.1N by exclusion of both exons 15 and 17D (Figure 6).

Mouse kidney 4.1B - 5'RACE experiments, using primer AP1 and a mouse 4.1B specific reverse primer (Table II), revealed that the predominant isoform of 4.1B in mouse kidney possessed a 5'structure distinct from the published cDNA from mouse brain (33). Whereas the brain form of 4.1B cDNA initiated translation at an ATG in a novel exon 2'-like region near the 5' end of the

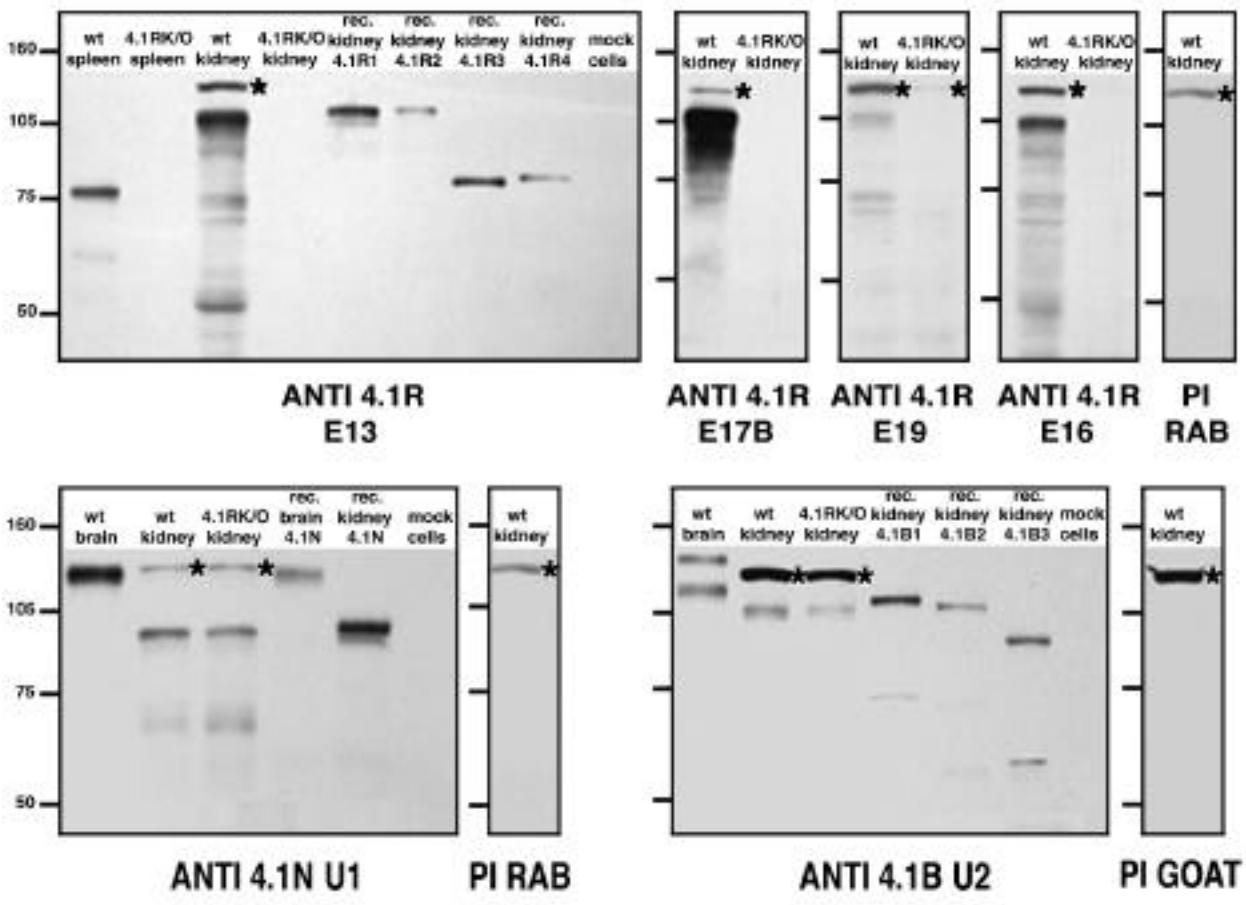
transcript, kidney 4.1B cDNAs were mostly spliced so as to delete this sequence. Therefore, kidney 4.1B was predicted to lack the N-terminal U1 region characteristic of brain 4.1B (33). We hypothesized that, like for 4.1R, translation of 4.1B might be initiated at a further downstream ATG site, whose position was analogous to ATG-2 in the 4.1R gene (Figure 1; 26,27). Transfection experiments with 4.1B expression plasmids confirmed this hypothesis (see below). 3' RACE experiments, using AP1 primer and a mouse 4.1B specific forward primer (Table II), confirmed that exon 21, a 117bp exon which encodes the last 36 amino acids of the coding region, the stop codon and the first 6bp of the 3'UTR region, was predominantly omitted in kidney (33). This resulted in a truncated spliceform including exon 20 followed by exon 22 which encodes a Glu residue and a stop codon. Amplification of full length mouse kidney 4.1B coding region, using a ATG-2 specific forward primer and a reverse primer annealing to exons 20 and 22, resulted in two PCR products, a major 2100bp product and a minor 1900bp product (Figure 5). Analysis of these products led to the characterization of three spliceforms. All three species showed concomitant exclusion of a 36bp exon and a 123bp exon, which we assigned to exon 15 and exon 17B, respectively (Figure 1). We previously reported exclusion of exon 15 (33). The major isoform lacked exons 15 and 17B. A second spliceform also excluded a 54bp stretch, corresponding to the beginning of exon 13, a splicing event previously reported in brain 4.1B (47). Further PCR screening using primers surrounding the spliced region in exon 13 showed that inclusion of this 54bp region was the more predominant event (data not shown). Because of the small difference in overall size between these two spliceforms, resulting from either inclusion or exclusion of the 54bp region in exon 13, they could not be visually discriminated within the 2100bp PCR product (Figure 5). A minor third spliceform lacked 192bp exon 17 in addition to partial skipping of exon 13 and to exclusion of exons 15 and 17B. This latter isoform was likely to correspond to the 1900bp PCR product (Figure 5), given the size of exon 17. Taken together, these data demonstrated that translation of the major kidney 4.1B isoform was initiated at an ATG-2 site, and that this spliceform lacked exons 15 and 17B and was terminated shortly after exon 20 (Figure 6). Another important spliceform also lacked a 54bp region in exon 13 (Figure 6).



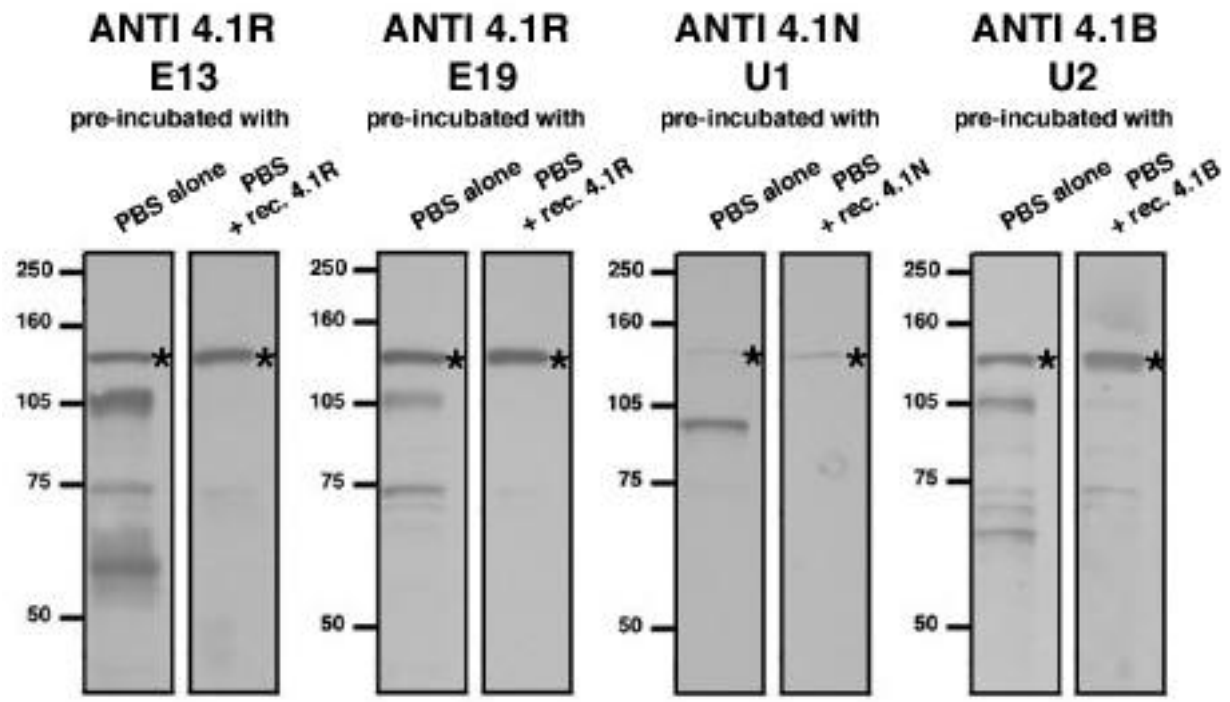
Mouse kidney 4.1G — Although 4.1G was not detected in immunohistochemistry experiments, we wanted to check first whether 4.1G cDNA species could be identified in kidney, and, second whether such cDNA structures, if any, could be detected or not as proteins by Western blotting. Neither 5'RACE nor 3'RACE experiments, using primer AP1 and either a mouse 4.1G specific reverse primer or a mouse 4.1G specific forward primer, respectively (Table II), revealed any splicing event which could affect translation at the very 5' end or 3' end of kidney 4.1G. Amplification of full length kidney mouse 4.1G coding region, using a forward primer corresponding to the beginning of mouse 4.1G coding region in exon 2 and a reverse primer specific for the end of mouse 4.1G coding region in exon 21, generated two PCR products of 2950bp and 2750bp, both expressed at similar levels (Figure 5). Sequencing of these PCR products

revealed that the 2950bp band corresponded to the prototypical mouse 4.1G isoform previously described (29), while the 2750bp band lacked both exons 16 and 17. Minor splicing events consisted of inclusion of exon 17C (1 out of 16 clones), and exclusion of exon 19 (1 out of 16 clones) (Figure 1). Taken together, these data indicated that the two major kidney 4.1G isoforms would be the prototypical isoform already described (29), and an isoform excluding both exons 16 and 17, a region encoding a SAB domain (48) similar to that described in 4.1R (49). However, these two species did not contribute to significant 4.1G protein expression in kidney (see previous section regarding immunohistochemistry and following section regarding Western Blot analysis).

Specific 4.1 proteins isoforms are expressed in kidney - The complexity of the structure of kidney 4.1 cDNAs was confirmed at the protein level. We probed whole kidney extracts with antibodies specific for each 4.1 protein (Figure 7). Whole spleen and brain extracts were used as "positive control tissues" for high level of expression of either 4.1R, or of 4.1N, 4.1G and 4.1B, respectively (Figure 7). The use of four 4.1R specific antibodies revealed that kidney 4.1R was expressed as a major 105kDa isoform, with minor isoforms migrating at ~70-75kDa (Figure 6, panel anti 4.1R E13-E16). The specificity of these bands as true protein 4.1R isoforms was supported by their absence in kidney extracts prepared from 4.1R null mice (Figure 7, panels anti 4.1R E13-E16). Moreover, the inability of anti 4.1R E13 and anti 4.1R E19 antibodies to detect any protein in 4.1R null kidney extracts supported that these antibodies were unable to interact with other 4.1 proteins than 4.1R, despite the weak cross-reactivity of anti 4.1R E13 antibody with recombinant 4.1N, 4.1B and 4.1G and that of anti 4.1R E19 antibody with recombinant 4.1B, respectively (Figure 2 and Table I). The size of the 105kDa isoform resembled that of the isoform previously described by us in human mammary epithelial cells (34), while the 75kDa isoforms were similar in size to the prototypical red cell 80kDa 4.1R (44). Blotting with a 4.1R antibody, raised against 4.1R unique region U1 confirmed the absence of this region in the 105kDa kidney 4.1R isoform (data not shown). As previously described (31), we confirmed that kidney 4.1N was a 95kDa protein (Figure 7, panel anti 4.1N U1). Importantly, anti 4.1N U1 antibody did not react with kidney 4.1R, despite the weak cross-reactivity of this antibody with recombinant 4.1R (Table I). It is also noteworthy that kidney 4.1R lacks the U1 region (Figure 6), the corresponding region in 4.1N used to raise the anti 4.1N U1 antibody (Figure 1). Protein 4.1B, previously reported to be expressed in kidney (47), was detected as a doublet of proteins migrating at 108kDa and 105kDa, respectively (Figure 7, panel anti 4.1B U2). Importantly, although these two kidney 4.1B isoforms were very similar in size to the major kidney 4.1R isoform, they were still expressed in 4.1R null kidney extract (Figure 7, panel anti 4.1B U2), emphasizing that they were truly the distinct 4.1B isoforms, an expected observation given the very high specificity of the anti 4.1B U2 antibody (Figure 2). Of particular note, all antibodies tested, including rabbit and goat preimmune antibodies (Figure 7, panels PI Rab and PI goat, respectively), showed a band migrating at ~130kDa in kidney (marked with an asterisk in all blots). This protein, detected in kidney (Figure 7, panels anti 4.1R E13-E16), was absent in spleen and brain (Figure 7, panel anti 4.1R E13 and panels anti 4.1N U1 and anti 4.1B U2, respectively). Importantly, this non specific band seen on Western blots did not confound immunohistochemical findings showing the complete lack of background staining in 4.1R null mice (see Figure 3). Protein 4.1G, probed with anti 4.1G U1 antibody (29), was barely detectable (data not shown), confirming our immunohistochemistry experiments. Importantly, the absence of 4.1G in kidney validated the use of anti 4.1R E16 antibody to study specific kidney 4.1R isoforms, the cross-reactivity of this 4.1R antibody with 4.1G not being an issue in kidney.



In order to assess further the specificity of detection of kidney 4.1 proteins and to definitely rule out that the 130kDa protein reacting with all antibodies tested in this study was a 4.1 isoform (Figure 7), wild type mouse whole kidney extracts were probed with four representative 4.1 antibodies, including the antibodies used for immunohistochemistry (anti 4.1R E13, anti 4.1R E19, anti 4.1N U1 and anti 4.1B U2), pre-adsorbed or not with either recombinant His-tagged full length mouse 4.1R (for anti 4.1R E13 and anti 4.1R E19 antibodies), 4.1N (for anti 4.1N U1 antibody) or 4.1B (for anti 4.1B U2 antibody). As expected, pre-incubation of 4.1 antibodies with PBS did not affect detection of renal 4.1 proteins in kidney extracts (Figure 8). In contrast, detection of kidney 4.1R was impaired by pre-incubation of anti 4.1R E13 and anti 4.1R E19 antibodies with recombinant 4.1R, that of kidney 4.1N by pre-incubation of anti 4.1N U1 antibody with recombinant 4.1N, and that of kidney 4.1B by pre-incubation of anti 4.1B U2 antibody with recombinant 4.1B (Figure 8). Importantly, unlike renal 4.1 proteins, detection of the 130kDa protein, marked with an asterisk, was not impaired by any of the pre-adsorption treatments tested here (Figure 8), supporting further our previous conclusion that this protein was not a 4.1 protein.



Major renal 4.1 protein isoforms can be assigned to cDNA species of known exon composition - Having characterized the major cDNA species amplified from mouse kidney marathon cDNA (Figure 6), we investigated whether these cDNA species, expressed as recombinant proteins in transfected COS-7 cells, would match the size of 4.1 proteins expressed in kidney. Because the recombinant 4.1 proteins were expressed with a C-terminal HA tag, their molecular weight was expected to be approximately 4kDa higher than that of the corresponding proteins expressed *in vivo* (33). The predicted predominant kidney 4.1R spliceform, initiated at ATG-2 translation site, lacking exon 16, and containing exon 17B (kidney 4.1R1), when expressed as a recombinant protein, closely matched the major 105kDa 4.1R isoform detected in kidney (Figure 7, panel anti 4.1R E13). Although the isoform, expressing both exons 16 and 17B (kidney 4.1R2), was predicted to be minor from our PCR screening, detection with our exon 16-encoded peptide antibody showed that this isoform was actually expressed at significant levels in kidney (Figure 7, panel anti 4.1R E16). The minor isoforms detected at ~75kDa were likely to be isoforms initiated at ATG-2, lacking exon 17B, and either excluding (kidney 4.1R3) or including (kidney 4.1R4) exon 16-encoded peptide (Figure 7, panel anti 4.1R E13). In contrast to their low level of expression in kidney, these ~75kDa isoforms were expressed at high levels in spleen (panel anti 4.1R E13). Expression of a cDNA encoding kidney 4.1N spliceform (i.e. lacking peptides encoded by exons 15 and 17D) resulted in a 95kDa protein migrating very similarly to the 4.1N isoform detected in kidney, while the 130kDa recombinant brain 4.1N isoform, was not detected in kidney (Figure 7, panel anti 4.1N U1), confirming a previous study (31). Two of the three recombinant kidney 4.1B proteins, one initiated at ATG-2 and lacking sequences encoded by exons 15, 17B, and 21 (kidney 4.1B1), and one also lacking 18 amino acids encoded by part of exon 13 (kidney 4.1B2), matched the protein expression profile observed in kidney, i.e a doublet of proteins migrating at 108kDa and 105kDa, respectively (Figure 7, panel anti 4.1B U2). By contrast, the third kidney 4.1B isoform identified by our cDNA screening, which also lacked exon 17-encoded peptide (kidney 4.1B3), did not have any counterpart *in vivo* (Figure 7, panel anti 4.1B U2).

Discussion

The present study provides a detailed description of the distribution of 4.1 proteins in kidney, at both the tissue and cellular level. Interestingly, despite the high sequence homology of key functional domains in 4.1 proteins, the proteins show discrete expression patterns along the nephron. We observed preferential accumulation of 4.1B in BC and PCT, of 4.1R in TAL, and of 4.1N in the thin limb of Henle's loop, DCT and all the regions of the collecting duct system. This likely reflects highly specific roles played by each 4.1 protein in renal structure and function, as has been previously reported for ankyrin (13-15). We do not identify 4.1G immunostaining in kidney, although it is plausible that kidney-specific 4.1G spliceforms lack the peptide sequences used to raise 4.1G antibodies. Our RACE experiments, however, do not identify 4.1G transcripts lacking such regions, further supporting the specific absence of 4.1G expression in kidney. In addition, others have reported the absence of 4.1G in kidney (47).

Based on morphological criteria suggesting a strong 4.1R gene promoter activity in the PCT region of the 4.1R null mouse nephron, we originally hypothesized that 4.1R would be preferentially expressed in PCT and likely participate in control of reabsorption (37). Based on our current results, the strong 4.1R gene promoter activity originally thought to be detected in PCT might actually be in TAL. Specific expression of 4.1R in TAL is consistent with a role for 4.1R in regulating reabsorption, since TAL is second to PCT in terms of reabsorption capacity (25% and 65% of overall nephron reabsorption, respectively). Interestingly, our preliminary studies have shown that the pH of urine collected from 4.1R null mice is significantly lower than the pH of urine collected from age matched wild type mice subject to a 48h food and water deprivation (Gascard P, Walensky L, Lee G, *et al*: *Mol Biol Cell* 10:154a, 1999, abstract). This observation suggests that the absence of 4.1R may alter function of transporters involved in maintenance of acid/base balance. We also found that 4.1N is expressed in collecting ducts. Collecting duct epithelium is made of two cell types. Intercalated cells participate in regulation of urine pH (50), while principal cells control water reabsorption through water channels, known as aquaporins (41). Here we report the presence of 4.1N in the baso-lateral membrane of both cell types. Interestingly, the anion exchanger AE1 has been shown to colocalize with ankyrin and spectrin in the baso-lateral region of intercalated cells (51). Moreover, it has been recently established that a mutation in kidney AE1 impairs its trafficking and targeting to the baso-lateral membrane, resulting in distal tubular acidosis (52). Given that 4.1N has been suggested to adopt also a vesicular distribution pattern in kidney (31), it is tempting to speculate that 4.1N, along with 4.1R, may participate, along with ankyrin and spectrin, in trafficking and anchorage of membrane transporters and, by doing so, may play a major role in maintaining urine characteristics, such as pH and volume.

4.1R interacts with red blood cell band 3, also known as anion exchanger isoform 1, AE1 (22-24), and binds *in vitro* to the cytoplasmic domain of sodium proton exchangers NHE1 and NHE2 (Sheryl Denker and Diane Barber, UCSF, San Francisco, CA, personal communication). Precedent in the published literature for interactions of proteins related to protein 4.1 with ion exchangers and transporters also exists. Ezrin, a member of the protein 4.1 superfamily (21), ensures proper targeting of NHE3 to the apical membrane through its interaction with EBP50 (6-11). The direct association of ezrin with NHE1 is critical for the restricted localization of NHE1 within lamellipodia in fibroblasts (12). Ankyrin, a cytoskeletal protein functionally related to 4.1R, interacts with various renal ion transporters to ensure their proper localization at the baso-lateral pole of the epithelium (19, 53-55). Thus, once sorted, membrane proteins are maintained in the appropriate location through interaction with cytoskeletal proteins such as ezrin, ankyrin and, possibly, protein 4.1. The importance of the cytoskeleton in maintaining kidney epithelial architecture is illustrated by the observation that renal ischemia, which results in proteolysis of ankyrin and spectrin, leads to a

major disorganization of kidney epithelial polarity (56). Interestingly, we have observed that red blood cells from 4.1R null mice showed a dramatic decrease in glycophorin C content (37), a transmembrane protein known to interact with 4.1R (57-60). 4.1N interacts with a subunit of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate glutamate receptor in brain, this interaction being necessary not only for proper targeting of the receptor but also for its level of expression (61). Thus, not only proper membrane localization but also level of expression of various membrane kidney transporters may depend on their interaction with kidney 4.1R, 4.1N and possibly 4.1B.

Restricted expression of 4.1B to BC suggests a potential role for this 4.1 protein in architecture and function of this region of the nephron, a very relevant finding given the frequent involvement of this structure in major nephropathies (62). Our preliminary observations suggest presence of 4.1B in parietal epithelial cells. Interestingly, parietal cells display different features than podocytes, the cells which surround the glomerulus, with respect to morphology, proliferation characteristics and expression of specific proteins (63, 64). Future work will further delineate 4.1B localization in BC.

The other highlight of the present study is a detailed characterization of the genetic structure of kidney 4.1R, kidney 4.1N, and kidney 4.1B. Our PCR experiments highlight kidney-specific splicing patterns for all 4.1 protein genes. Translation of all kidney 4.1R isoforms is exclusively initiated at the ATG-2 site, with these isoforms specifically lacking the U1 region. This finding is functionally relevant as we have implicated this region in modulating 4.1R interactions with binding partners (unpublished data). The major kidney 4.1R isoform excludes exon 16, which encodes both a key component of the SAB domain (39,49) and a nuclear localization signal (25). This isoform also contains exon 17B-encoded peptide, a splicing event restricted to epithelial tissues (34). 4.1N isoforms also exhibit tissue specificity. A 95kDa 4.1N isoform is expressed in kidney, whereas a 130kDa isoform is detected in brain (31). Evaluation of the functional impact of the absence of the two sequences encoded by exons 15 and 17D in kidney 4.1N warrants further study. 4.1B also displays kidney-specific splicing events. First, we report for the first time exclusion of a novel exon, similar to exon 2' in 4.1R gene (26), containing the ATG translation initiation site. Initiation of kidney 4.1B translation at a downstream ATG site, resulting in the omission of the U1 region, may be as functionally relevant to 4.1B as it is to 4.1R. Second, we describe exclusion of exon 17B. Last, we confirm exclusion of exons 15 and 21 (33). Future work will investigate the structural and functional impact of the absence of these various peptides in kidney 4.1 proteins.

We conclude that members of the protein 4.1 gene family are only expressed in distinct regions of the nephron but also show differential subcellular distribution. We speculate that 4.1 proteins, like ankyrins, may play unique roles in renal structure and function. Future investigation of the roles of 4.1 proteins in kidney function will be based on the characterization of renal 4.1 isoforms and the detailed analysis of their tissue distribution presented here. Identification of specific binding partners for each kidney 4.1 protein, in addition to the study of possible perturbations in renal function in a panel of 4.1 knock out mice, will enable us to further define the functional relevance of 4.1 proteins in kidney.

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Table I. Assessment of antibody specificity against 4.1 recombinant proteins. Antibodies against recombinant proteins or peptides encoding unique regions of mouse 4.1R, 4.1G, 4.1N and 4.1B, were tested against 100ng of full length recombinant mouse 4.1R, initiated at ATG-1 and expressing exon 17B-encoded peptide, 4.1G (30), 4.1N (32) and 4.1B (34) as described in the Methods section. Each antibody reacted strongly with its corresponding protein. Some antibodies reacted at various extents with the three other 4.1 proteins. We want to emphasize that most weak cross-reactivities listed in this Table do not appear on the Western Blots presented in Figure 2. This is because such weak signals can only be detected after long exposure of the blots leading to saturation of the signal obtained with the protein reacting strongly with the antibody. Therefore, as extensively shown in this study, these weak cross-reactivities (labeled +/-) should not be considered as very relevant. The following grading system was based on immunoblot reactivity: ++ : strong reactivity; + : significant reactivity; +/- : weak reactivity; 0 : no reactivity.

	mouse 4.1R	**mouse 4.1N**	**mouse 4.1B**	**mouse 4.1G**
Anti 4.1R U1 region (RU1)	++	0	0	0
Anti 4.1R exon 13 (RE13)	++	+/-	+/-	+/-
Anti 4.1R exon 16 (RE16)	++	0	0	++
Anti 4.1R exon 17B (RE17B)	++	0	0	0
Anti 4.1R exon 19 (RE19)	++	0	+/-	0
Anti 4.1R exon 21 (RE21)	++	+	+	++
Anti 4.1N U1 region (NU1)	+/-	++	+/-	+/-
Anti 4.1B peptide exon 13 (BU2)	0	0	++	0
Anti 4.1G U1 region (GU1)	0	0	0	++
Anti 4.1G U3 region (GU3)	++	++	++	++

Table II. List of 4.1 gene specific primers used for 5'RACE and 3'RACE experiments. Features of 4.1 gene specific reverse primers used for 5'RACE and forward primers used for 3'RACE experiments are presented. Primers were optimized using MacVector software version 6.0 (Oxford Molecular Ltd., Oxford, England), i.e. selected in particular for a high melting temperature (>65°C) in order to minimize non specific PCR amplification of mouse kidney marathon cDNA.

Gene	Type of experiment	Primer sequence	Exon location
4.1R	5'RACE	5'-AATGTTCTTGCTCTCCAGGCCGGAT	exons 11-10
4.1R	3'RACE	5'-GCAGCAGCTTCTTCATCAAGATCCG	exon 10
4.1G	5'RACE	5'-TCCACACACACCTTCCATAGCCGTT	exon 11
4.1G	3'RACE	5'-CACCACAATTTGAGCGTGCCTCTAGT	exon 12
4.1N	5'RACE	5'-TCAAGGCTGCGGGACATGGTATAGC	exon 12
4.1N	3'RACE	5'-CCTCAGTCAGTGAGAATCACGATGC	exon 13
4.1B	5'RACE	5'-GAGAGATGCCCTTTGTTGTGGCGTA	exon 13
4.1B	3'RACE	5'-AGAAAAAGGCTGAAGAGGAGCGCGT	exon 13

Figure legends

Figure 1. Exon maps of mouse 4.1 protein genes and corresponding protein domains. Maps show exon composition and corresponding protein domains for the four mouse 4.1 genes, 4.1R, 4.1G, 4.1N, and 4.1B (maps for mouse 4.1N and 4.1B genes are derived from corresponding human genes). Exons in grey are constitutive, those in black alternative, and those in white non coding. Three conserved protein domains, the membrane binding domain (MBD), the spectrin-actin binding domain (SAB), and the C-terminal domain (CTD), and the corresponding regions in 4.1G, 4.1N and 4.1B with percentage of homology with 4.1R, are displayed in white. The SAB domain is non conserved (nc) in 4.1N. The regions in grey, interspersed with conserved domains, are poorly conserved among 4.1 proteins (unique regions U1, U2, and U3). Note that the maps display all exons known so far for each 4.1 gene, regardless of their tissue-specific expression. Some exons, in particular in the unique U2 and U3 regions and in the SAB domain, may be omitted in the tissues that we referred to in the present study, i.e. brain, spleen or kidney. The figure also shows location of the peptides used to raise antibodies specific for each protein 4.1 gene product: RU1, RE13, RE16, RE17B, RE19 and RE21 for 4.1R; GU1 and GU3 for 4.1G; NU1 for 4.1N; and BU2 for 4.1B.

Figure 2. Specificity of 4.1 protein antibodies assessed by reactivity with recombinant His-tagged full length mouse proteins 4.1R, 4.1N, 4.1B and 4.1G. Antibodies raised against recombinant proteins or peptides encoding various regions of human 4.1R (anti 4.1R U1, anti 4.1R E16, anti 4.1R E19 and anti 4.1R E21) or mouse 4.1R (anti 4.1R E13 and anti 4.1R E17B), mouse

4.1G (anti 4.1G U1 and anti 4.1GU3), mouse 4.1N (anti 4.1N U1) and mouse 4.1B (anti 4.1B U2), were probed against 100ng of recombinant His-tagged full length mouse proteins 4.1R, 4.1N, 4.1B and 4.1G as described in the Methods section. Equal loading of recombinant proteins was confirmed after protein assay and staining of a gel with Gelcode® blue stain reagent. Most antibodies reacted predominantly with their corresponding 4.1 protein. Three antibodies showed strong cross-reactivity: anti 4.1R E16 antibody with 4.1G, anti 4.1R E21 antibody with 4.1N, 4.1B and 4.1G, and anti 4.1G U3 antibody with 4.1R, 4.1N and 4.1B, respectively. Three antibodies showed weak cross-reactivity: anti 4.1R E13 antibody with 4.1N, 4.1B and 4.1G, anti 4.1R E19 antibody with 4.1B and anti 4.1N U1 antibody with 4.1R, 4.1B and 4.1G, respectively (Table I). Such weak signals could only be detected after long exposure of the blots leading to a saturation of the signal obtained with the protein reacting strongly with the antibody (data not shown). Therefore, as extensively shown in this study, these weak cross-reactivities should not be considered as very relevant. Molecular weight protein markers expressed in kDa are shown on the left of each panel. The information gathered through this Western Blot analysis is summarized in Table I.

Figure 3. Tissue distribution of 4.1 proteins in kidney. Wild type or 4.1R null mouse kidney sections were stained with antibodies specific for each 4.1 protein as described in the Methods section. A: kidney sections were stained with either anti 4.1R E19 antibody (first row), anti 4.1N U1 antibody (second row), or anti 4.1B U2 antibody (third row). As expected from 4.1 protein distribution in wild type kidney, 4.1R showed no signal in any 4.1R null kidney section (shown only for outer medulla), while 4.1N showed signal in all 4.1R null sections (shown only for outer medulla), and 4.1B only in cortical 4.1R null sections. Insets in left panel of first and second row highlight baso-lateral expression of 4.1R and 4.1N, respectively. Nuclei (in green) were stained with sytox in these sections. Insets in first left panel of third row highlight 4.1B diffuse expression in BC (upper inset) and apical expression in some PCTs (lower inset). B: wild type mouse kidney sections were stained with anti protein Tamm-Horsfall antibody, a specific marker for TAL, and either anti 4.1R E19 antibody (two panels from the left) or anti 4.1N U1 antibody (right panel). C: wild type mouse kidney sections were stained with anti aquaporin-2 antibody, a specific marker for collecting duct, and either anti 4.1R E19 antibody (left panel) or anti 4.1N U1 antibody (two panels from the right). BC: Bowman's capsule; CCD: cortical collecting duct; cTAL: cortical thick ascending limb of Henle's loop; IMCD: inner medullary collecting duct; mTAL: medullary thick ascending limb of Henle's loop; OMCD: outer medullary collecting duct; PCT: proximal convoluted tubule. Scale bar: 100μm.

Figure 4. Distribution of 4.1 proteins along the nephron. The schematic diagram displays identity of renal 4.1 proteins and their distribution along the nephron: 4.1B in BC and PCT, 4.1R in TAL, 4.1N in thin limb of Henle's loop, DCT, CCD, OMCD and IMCD. Because the 4.1N antibody does not stain all thin limb sections in kidney sections, and in the absence of specific markers for either the thin descending limb or the thin ascending limb, we cannot conclude which of these two structures 4.1N is expressed in.

Figure 5. Characterization of kidney-specific protein 4.1 cDNA spliceoforms. Full length 4.1 protein coding regions were amplified from mouse kidney marathon cDNA using pairs of primers specific for each 4.1 gene (see Methods section). Location of primers used for PCR amplification and resulting PCR products are shown for each 4.1 gene. Size of each PCR product expressed in base pair (bp) is displayed.

Figure 6. Maps of major protein 4.1 kidney spliceoforms. Exonic map and protein structure is displayed for each major renal 4.1 isoform identified by PCR screening: 105kDa kidney 4.1R, 95kDa kidney 4.1N, 108 and 105kDa kidney 4.1Bs. The 108kDa 4.1B isoform expresses the 18 amino acids encoded by the 54bp region shown in black in exon 13, while the 105kDa isoform does not. Constitutive exons are shown in grey, alternative exons in black and non coding exons in white.

Figure 7. Characterization of renal 4.1 proteins in mouse whole kidney extracts. Western blot analysis of mouse kidney, spleen or brain extracts (60μg total protein) and of lysates of COS-7 cells transfected with cDNAs encoding HA epitope-tagged mouse kidney 4.1R, 4.1N or 4.1B spliceoforms was carried out as described in the Methods section. The 4.1R and 4.1B recombinant proteins displayed on the Western blot, all initiating at their respective ATG-2 site, are as follows: kidney 4.1R1: 4.1R lacking exon 16 and containing exon 17B-encoded peptides (major kidney 4.1R isoform); kidney 4.1R2: 4.1R containing exon 16 and exon 17B-encoded peptides; kidney 4.1R3: 4.1R lacking exon 16 and exon 17B-encoded peptides; kidney 4.1R4: 4.1R containing exon 16 and lacking exon 17B-encoded peptides (red cell 4.1R); kidney 4.1B1: 4.1B lacking exon 15, exon 17B, and exon 21-encoded peptides (major kidney 4.1B isoform); kidney 4.1B2: 4.1B lacking 54bp exon 13, exon 15, exon 17B, and exon 21-encoded peptides; kidney 4.1B3: lacking 54bp exon 13, exon 15, exon 17, exon 17B, and exon 21-encoded peptides. All antibodies tested, including rabbit and goat preimmune antibodies (PI rab and PI goat), detected a non specific band in wild type kidney extract (marked with an asterisk on the right side of the bands). Molecular weight markers expressed in kDa are shown on the left side of each panel.

Figure 8. Specificity of detection of renal 4.1 proteins in mouse whole kidney extracts. Wild type mouse whole kidney lysates were probed with anti 4.1R E13, anti 4.1R E19, anti 4.1N U1, or anti 4.1B U2 antibodies pre-incubated with either PBS or PBS containing a 10 molar excess of either recombinant His-tagged full length mouse 4.1R, 4.1N or 4.1B, as described in the Methods section. As expected, detection of each renal 4.1 protein was ablated when the antibody used to probe the membrane was pre-adsorbed with the corresponding recombinant mouse 4.1 protein. Importantly, detection of the 130kDa protein, marked with an asterisk, was not affected by any of the pre-adsorption treatments, emphasizing that this protein was not a renal 4.1 protein. Molecular weight protein markers expressed in kDa are shown on the left of each panel.